EXHIBIT A

Declaration of Barbara Froesch Ph.D. regarding amendment to the Specification

Appendix 1 – CV of Barbara Froesch Ph.D.

Appendix 2 – Kay, et al., *Protein Sci.* 8: 435-438 (1999)

Appendix 3 – Rosenthal et al., *J Biol Chem* 274(48): 33959-33964 (1999)

Docket No.: 27656/38365A
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Ernst Hafen et al.

Application No.: 10/509,558 Confirmation No.: 1027

Filed: March 25, 2005 Art Unit: 1649

For: GROWTH REGULATING PROTEINS Examiner: G. S. Emch

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Barbara Froesch, declare the following:

1. I graduated in 1992 from the Swiss Federal Technology Institute (ETH) in Zurich, where I studied pharmaceutical sciences. After the Ph.D. work in the Department of Oncology at the University Hospital of Zurich, I dedicated three years to the study of tumor biology in Dr. John Reed' s laboratory at The Burnham Institute in La Jolla, USA. I joined The Genetics Company, a spin-off of the University of Zurich and the ISREC of Epalinges, as a research scientist in January 1999 and I am currently head of the Biology Department and program manager for cancer therapeutics.

2. I submit this declaration to address facts relating to the discovery of a typographical error in the above-identified application as filed to the sequence of the Epsin NH_2-terminal Homology (ENTH) domain as listed in the specification at page 2. Specifically, the sequence of the ENTH domain provided at page 2 of the specification contains errors at three (3) locations which would have been obvious from an examination of the reference cited as the source of the ENTH domain (Kay et al., *Protein Sci.* 8: 435-438

(1999); Appendix 2), the reference cited as the source of the EPSIN protein (Rosenthal et al., *J Biol Chem* 274(48): 33959-33964 (1999); Appendix 3), as well as in the sequence listings of the application itself.

3. In reading the specification and sequence listing of the application, one of ordinary skill in the art would have recognized an internal discrepancy in the application as filed between the summary sequence of the ENTH domain at page 2, lines 29-31, of the specification and the actual sequences as presented in Seq. ID Nos 1, 2, 3, 4, and 5. This inconsistency is best viewed by an alignment of the sequences, as shown below. The alignment also includes the sequences of the ENTH domains of EPSIN proteins from the Rosenthal et al. reference and the consensus sequence of ENTH domains from the Kay et al. reference, where the number in parentheses is the number of the amino acid residue that begins the ENTH domain in each sequence.

ENTH Domain (as filed)	N- X(11-13) -V- X2-A-T- X(34-36)-
ENTH Domain (as corrected)	N- X(11-13) -V- X2-A-T- X(34-36)-
SEQ ID No 1	(16)N-V-V-M-N-Y-S-E-I-E-S-K-V-R-E-A-T-N-D-D-P-W-G-P-S-G-Q-L-M-G-E-I-A-K-A-T-F-M-Y-
SEQ ID No 2	(16)N-V-V-M-N-Y-S-E-I-E-S-K-V-R-E-A-T-N-D-D-P-W-G-P-S-G-Q-L-M-G-E-I-A-K-A-T-F-M-Y-
SEQ ID No 3	(20)N-V-V-M-N-Y-T-E-T-E-G-K-V-R-E-A-T-N-D-D-P-W-G-P-T-G-P-L-M-Q-E-L-A-Y-S-T-F-S-Y-
SEQ ID No 4	(20)N-V-V-M-N-Y-T-E-T-E-G-K-V-R-E-A-T-N-D-D-P-W-G-P-T-G-P-L-M-Q-E-L-A-Y-S-T-F-S-Y-
SEQ ID No 5	(16)N-V-V-M-N-Y-S-E-I-E-S-K-V-R-E-A-T-N-D-D-P-W-G-P-S-G-Q-L-M-G-E-I-A-K-A-T-F-M-Y-
Rosenthal et al. FIG 1A rEPSIN2a	(12)N-I-V-N-N-Y-S-E-A-E-I-K-V-R-E-A-T-S-N-D-P-W-G-P-S-S-S-L-M-T-E-I-A-D-L-T-Y-N-V-
Rosenthal et al. FIG 1A rEPSIN2	(12)N-I-V-N-N-Y-S-E-A-E-I-K-V-R-E-A-T-S-N-D-P-W-G-P-S-S-S-L-M-T-E-I-A-D-L-T-Y-N-V-
Rosenthal et al. FIG 1A rEPSIN1	(12)N-I-V-H-N-Y-S-E-A-E-I-K-V-R-E-A-T-S-N-D-P-W-G-P-S-S-S-L-M-S-E-I-A-D-L-T-Y-N-V-
Kay et al. FIG 1 consensus sequence	N- X(11-13) -V- X2-A-T- X(34-36)-

ENTH Domain (as filed)	-R- X(7-8) -W-R- X3 -K- *X12*
ENTH Domain (as corrected)	-R- X(7-8) -W-R- X3 -K- *X11*
SEQ ID No 1	-E-Q-F-P-E-L-M-N-M-L-W-S-R-M-L-K-D-N-K-K-N-W-R-R-V-Y-K-S-L-L-L-L-A-Y-L-I-R-N-
SEQ ID No 2	-E-Q-F-P-E-L-M-N-M-L-W-S-R-M-L-K-D-N-K-K-N-W-R-R-V-Y-K-S-L-L-L-L-A-W-L-I-R-N-
SEQ ID No 3	-E-T-F-P-E-V-M-S-M-L-W-K-R-M-L-Q-D-N-K-T-N-W-R-R-T-Y-K-S-L-L-L-L-N-Y-L-V-R-N-
SEQ ID No 4	-E-T-F-P-E-V-M-S-M-L-W-K-R-M-L-Q-D-N-K-T-N-W-R-R-T-Y-K-S-L-L-L-L-N-Y-L-V-R-N-
SEQ ID No 5	-E-Q-F-P-E-L-M-N-M-L-W-S-R-M-L-K-D-N-K-K-N-W-R-R-V-Y-K-S-L-L-L-L-A-Y-L-I-R-N-
Rosenthal et al. FIG 1A rEPSIN2a	-V-A-F-S-E-I-M-S-M-V-W-K-R-L-N-D-H-G-K-N- W-R-H-V-Y-K-A-L-T-L-L-D-Y-L-I-K-T-
Rosenthal et al. FIG 1A rEPSIN2	-V-A-F-S-E-I-M-S-M-V-W-K-R-L-N-D-H-G-K-N- W-R-H-V-Y-K-A-L-T-L-L-D-Y-L-I-K-T-
Rosenthal et al. FIG 1A rEPSIN1	-V-A-F-S-E-I-M-S-M-I-W-K-R-L-N-D-H-G-K-N- W-R-H-V-Y-K-A-M-T-L-M-E-Y-L-I-K-T-
Kay et al. FIG 1 consensus sequence	-R- X(7-8) -W-R- X3 -K- X11

ENTH Domain (as filed)	-G-X-E- X15 -L- *X(11-12)-* D-X-G-*(null)*R-
ENTH Domain (as corrected)	-G-X-E- X15 -L- *X(10-11)-* D-X-G- *X3* -R-
SEQ ID No 1	-G-S-E-R-V-V-T-S-A-R-E-H-I-Y-D-L-R-S-L-E-N-Y-H-F-V-D-E-H-G-K-D-Q-G-I-N-I-R-Q-K-
SEQ ID No 2	-G-S-E-R-V-V-T-S-A-R-E-H-I-Y-D-L-R-S-L-E-N-Y-H-F-V-D-E-H-G-K-D-Q-G-I-N-I-R-Q-K-
SEQ ID No 3	-G-S-E-R-V-V-T-S-S-R-E-H-I-Y-D-L-R-S-L-E-N-Y-T-F-T-D-E-G-G-K-D-Q-G-I-N-V-R-H-K-
SEQ ID No 4	-G-S-E-R-V-V-T-S-S-R-E-H-I-Y-D-L-R-S-L-E-N-Y-T-F-T-D-E-G-G-K-D-Q-G-I-N-V-R-H-K-
SEQ ID No 5	-G-S-E-R-V-V-T-S-A-R-E-H-I-Y-D-L-R-S-L-E-N-Y-H-F-V-D-E-H-G-K-D-Q-G-I-N-I-R-Q-K-
Rosenthal et al. FIG 1A rEPSIN2a	-G-S-E-R-V-A-Q-D-C-R-E-N-I-F-A-I-Q-T-L-K-D-F-Q-Y-I-D-R-D-G-K-D-Q-G-I-N-V-R-E-K-
Rosenthal et al. FIG 1A rEPSIN2	-G-S-E-R-V-A-Q-D-C-R-E-N-I-F-A-I-Q-T-L-K-D-F-Q-Y-I-D-R-D-G-K-D-Q-G-I-N-V-R-E-K-
Rosenthal et al. FIG 1A rEPSIN1	-G-S-E-R-V-S-Q-D-C-K-E-N-M-Y-A-V-Q-T-L-K-D-F-Q-Y-V-D-R-D-G-K-D-Q-G-V-N-V-R-E-K-
Kay et al. FIG 1 consensus sequence	-G-X-E- X15 -L- X(10-11)- D-X-G- X3 -R-

ENTH Domain (as filed)	X11- D- X7 -R-...
ENTH Domain (as corrected)	X11- D- X7 -R-...
SEQ ID No 1	-V-K-E-L-V-E-F-A-Q-D-D-D-R-L-R-E-E-R-...
SEQ ID No 2	-V-K-E-L-V-E-F-A-Q-D-D-D-R-L-R-E-E-R-...
SEQ ID No 3	-V-R-E-L-I-D-F-I-Q-D-D-D-R-L-R-E-E-R-...
SEQ ID No 4	-V-R-E-L-I-D-F-I-Q-D-D-D-R-L-R-E-E-R-...
SEQ ID No 5	-V-K-E-L-V-E-F-A-Q-D-D-D-R-L-R-E-E-R-...
Rosenthal et al. FIG 1A rEPSIN2a	-S-K-D-L-V-A-L-L-K-D-E-E-R-L-K-A-E-R-...
Rosenthal et al. FIG 1A rEPSIN2	-S-K-D-L-V-A-L-L-K-D-E-E-R-L-K-V-E-R-...
Rosenthal et al. FIG 1A rEPSIN1	-A-K-D-L-V-A-L-L-R-D-E-D-R-L-R-E-E-R-...
Kay et al. FIG 1 consensus sequence	X11- D- X7 -R-...

4. In order to reconcile this discrepancy, one of skill in the art would have consulted the Kay reference cited in the specification at page 6, lines 33-34 (fully cited at page 39, lines 32-35) concerning the ENTH domain and the Rosenthal reference cited in the specification at page 6, line 35 (fully cited at page 41, lines 13-16) concerning EPSIN proteins. The Kay reference contains the same summary sequence in its abstract (Appendix 1, p 435) and in its text (Appendix 1, p 436, column 1, lines 3-4 and p 437, column 1, lines 2-3) as that in the specification of the present application. However, the person of skill in the art would have recognized the definitive source of the summary sequence as the analysis of the primary sequences of 11 proteins containing ENTH domains (Appendix 1, Figure 1), where the sequences of the 11 proteins are aligned, and the spacing between the evolutionarily conserved residues is accurately presented in the consensus sequence, giving the correct summary sequence of the ENTH domain. In addition, the person of skill in the art would have recognized that the ENTH domains of the EPSIN proteins of the Rosenthal reference are consistent with the consensus sequence of the Kay reference (see alignments above and Appendix 2, Fig 1A), and recognized that the ENTH domain summary sequence was a simple typographical error.

5. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001.

Dated: December 15, 2005

B Froesch

Barbara Froesch

Appendix 1 – CV of Barbara Froesch Ph.D.

Curriculum Vitae

Barbara Alessandra FROESCH, Ph.D.
Weidstrasse 25
8932 Mettmenstetten

DATE OF BIRTH: May, 10th 1968

NATIONALITY: Swiss

EDUCATION: 1/1996: Doctoral degree ETH in natural sciences

1993-1995: Ph.D. training at the Division of Oncology of the University Hospital, Zurich, CH. Thesis: "Combination of cytotoxic immunoconjugates and molecular intervention in *bcl-2* oncogene expression to overcome multidrug resistance in small cell lung cancer". Supervisors: Rolf Stahel, MD, Professor at the University Hospital of Zurich and Gerd Folkers, Ph.D., Professor of Pharmaceutical Chemistry at the ETH Zurich.

1992: Swiss Federal Master in Pharmaceutical Sciences ETH.

1987-1992: Study of Pharmaceutical Sciences at the Swiss Federal Institute of Technology (ETH), Zurich, CH.

EMPLOYMENT: Since 1/2004: **Department Head Biology,** The Genetics Company, Zurich, CH

Since 1/2002: **Program Manager Cancer Therapeutics**, The Genetics Company, Zurich, CH

2001-2004: **Intellectual Property Manager,** The Genetics Company, Zurich

1999-2001: **Scientist**, The Genetics Company, Zurich, CH.

March 1996-January 1999: **Postdoctoral fellow**, The Burnham Institute, La Jolla, CA, USA. "Role of the Bcl-2-associated BAG-1 proteins in prostate cancer", and "Regulation of p53 transcriptional activity in chemoresistant cancer".

Supervisor: John C. Reed, M.D., Ph.D.

1993-1996: **Pharmacist** (part-time employment), Aponova Apotheke, Glattzentrum, Wallisellen

1991-1992: **Pharmacist-assistant** (part-time emplyment), several swiss pharmacies.

1990-1991: **Practical training in a pharmacy,** Lintheschergasse-Apotheke, Zurich, CH. Supervisor: K. Egloff, Ph.D.

LANGUAGES Italian: native language

German, French, English: very good knowledge (oral and written)

List of Publications

Hafen E., Schütt, C., **Froesch, B.** Drosophila - a model system for targets and lead identification in cancer and metabolic disorders. In *Model Organisms in Drug Discovery, Pamela Carroll and Kevin Fitzgerald, editors. In Press*

Kramps T.*, Peter O.*, Brunner E.*, Nellen D., **Froesch B.**, Chatterjee S., Murone M., Zullig S., and Basler K. Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell 109(1):47-60, 2002.*

Bentires-Alj M., Dejardin E., Viatour P., Van Lint C., **Froesch B.**, Reed J. C., Merville M. P., and Bours V. Inhibition of the NF-kappa B transcription factor increases Bax expression in cancer cell lines. *Oncogene 20(22):2805-13, 2001.*

Froesch B.A.*, Knee D.A.*, Nuber U., Takayama S., Reed J.C. Structure-function analysis of Bag1 proteins. Effects on androgen receptor transcriptional activity. *J. Biol. Chem. 276(16)*:12718-24, 2001.

Froesch, B.A., Aime-Sempe, C., Leber, B., Andrews, D., and Reed, J.C. Inhibition of p53 transcriptional activity by Bcl-2 requires its membrane anchoring domain, *J. Biol. Chem. 274: 6469-6475, 1999.*

Froesch, B.A., Takayama, S., and Reed, J. C. BAG-1L protein enhances androgen receptor function. *J. Biol. Chem. 273: 11660-11666, 1998.*

Matsuzawa, S., Takayama, S., **Froesch, B. A.**, Zapata, J. M., and Reed, J. C. p53-Inducible human homologue of Drosophila seven absentia (Siah) inhibits cell growth: suppression by BAG-1, *EMBO J. 17 (10):2736-2747, 1998.*

Liu, R., Takayama, S., Zheng, Y., **Froesch, B.**, Chen, G.O., Zhang, X., Reed, J.C. and Zhang, X.K. Interaction of BAG-1 with retinoic acid receptor and its inhibition of retinoic acid-induced apoptosis in cancer cells. *J. Biol. Chem. 273(27):16985-92, 1998.*

Zimmermann, S., Wels, W., **Froesch, B. A.**, Gerstmayer, B., Stahel, R. A., Zangemeister-Wottke, U. A novel immunotoxin recognising the epithelial glycoprotein-2 has potent antitumoral activity on chemotherapy-resistant lung cancer, *Cancer Immunology Immunotherapy 44: 1-9, 1997.*

Froesch, B. A., Luedke G. H., Ziegler, A., Stahel, R. A. & Zangemeister-Wittke, U. The synergistic effect of a doxorubicin immunoconjugate and *bcl-2* antisense oligonucleotides on non-resistant and drug resistant small cell lung cancer cell lines, *Tumor Targeting 2: 265-278, 1996.*

Froesch, B. A., Stahel, R. A. & Zangemeister-Wittke, U., Preparation and functional evaluation of new doxorubicin immunoconjugates containing an acid sensitive linker on small cell lung cancer cells, Cancer Immunology Immunotherapy: 42: 55-60, 1996.

Zangemeister-Wittke, U., Collison, A. R., **Frösch, B.**, Waibel, R., Schenker, T. and Stahel, R. A., Immunotoxins recognising a new epitope on the neural cell adhesion molecule have potent cytotoxic effects against small cell lung cancer, *Br. J. Cancer 69, 32-39 , 1994.*

Patent applications

Basler, K., Brunner, E., **Froesch, B.**, Kramps, T., Peter, O. Essential downstream component of the wingless signaling pathway and therapeutic and diagnostic applications based thereon. *Publication number: US2001000915543*

Pof

List of Presentations at Scientific Meetings

Froesch, B. A. "From Startup toward fully integrated drug development company" Wokshop organized by Maybridge, Bregenz, Austria 2003 (invited speaker)

Froesch, B. A., From a disease model to a potential therapy for colon cancer. *First European conference on cell-based assays & high content screening, September 2003, London UK (Invited speaker)*

Froesch, B. A. From the fruit fly to a potential therapy for colon cancer. *MipTec 2003, Basel, Switzerland (Invited speaker)*

Froesch, B. A., From a disease model to a potential therapy for colon cancer. *Novel approaches for the discovery of anticancer agents, Freiburg, Germany, June 2003 (Poster discussion session)*

Froesch, B. A., Aimé-Sempé, C., Leber, B., Andrews, D., and Reed, J. C. Inhibition of p53 transcriptional activity by Bcl-2 requires its membrane anchoring domain. *90th Annual Meeting of the American Association for Cancer Research, April 1999, Philadelphia (poster discussion session)*

Froesch, B. A., Takayama, S., and Reed, J. C., BAG-1L protein enhances androgen receptor function, *Keystone Symposia, March 1998, Lake Tahoe (poster)*

Froesch, B. A., Krajewska, M., Takayama, S., Kitada, S., and Reed, J. C., BAG-1L protein is expressed in prostate cancers and enhances androgen receptor function, *89th Annual Meeting of the American Association for Cancer Research, March 1998, New Orleans (poster)*

Froesch, B. A. & Zangemeister-Wittke, U., Combined application of bcl-2 antisense and a selective doxorubicin-immunoconjugate to overcome drug resistance in small cell lung cancer, *Annual Meeting of the Swiss Society for Oncology and Dr. Josef Steiner Cancer Prize Award, November 1995, Bern (oral presentation)*

Froesch, B. A., Stahel, R. A. and Zangemeister-Wittke, U. Combination of doxorubicin-immunoconjugates and molecular intervention in bcl-2 oncogene expression to overcome drug resistance in small cell lung cancer, *86th Annual Meeting of the American Association for Cancer Research, March 1995, Toronto, Canada (oral presentation).*

Froesch, B. A., Zangemeister-Wittke, U., Rose, K. and Stahel, R. A., Cytotoxic activity of doxorubicin- and ETA42-immunoconjugates against drug resistant small cell lung cancer cell lines in vitro, *1st International Conference on Reversal of Multidrug Resistance in Cancer, St. Gallen, Switzerland, September 1994 (poster).*

Awards and Fellowships

Dr. Arnold and Susanne Huggenberger-Bishoff Award for Cancer Research (October 1995)

Swiss National Foundation (Schweizerischer Nationalfonds): Fellowship (March 1996 - February 1997)

Cancer Research Switzerland (Krebsforschung Schweiz): Fellowship (March 1997 - February 1998)

Schweiz. Stiftung fuer medizinisch-biologische Stipendien: Fellowship (March 1998- February 1999)

AFLAC Travel Award for Cancer Research (February 1999)

Polypops Award for the best presentation (MipTec Basel 2003)

Appendix 2 – Kay, et al., *Protein Sci.* 8: 435-438 (1999)

Protein Science (1999), 8:435–438. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Identification of a novel domain shared by putative components of the endocytic and cytoskeletal machinery

BRIAN K. KAY,[1] MONTAROP YAMABHAI,[1] BEVERLY WENDLAND,[2] AND SCOTT D. EMR[3]

[1]Department of Pharmacology, University of Wisconsin-Madison, Madison, Wisconsin 53706-1532
[2]Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218
[3]Division of Cellular and Molecular Medicine and Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, La Jolla, California 92093-0668

(RECEIVED August 18, 1998; ACCEPTED October 22, 1998)

Abstract: We have identified a ~140 amino acid domain that is shared by a variety of proteins in budding and fission yeast, nematode, rat, mouse, frog, oat, and man. Typically, this domain is located within 20 residues of the N-terminus of the various proteins. The percent identity among the domains in the 12 proteins ranges from 42 to 93%, with 16 absolutely conserved residues: $N\text{-}x_{11-13}\text{-}V\text{-}x_2\text{-}A\text{-}T\text{-}x_{34-36}\text{-}R\text{-}x_{7-8}\text{-}W\text{-}R\text{-}x_3\text{-}K\text{-}x_{12}\text{-}G\text{-}x\text{-}E\text{-}x_{15}\text{-}L\text{-}x_{11-12}\text{-}D\text{-}x\text{-}G\text{-}R\text{-}x_{11}\text{-}D\text{-}x_7\text{-}R$. Even though these proteins share little beyond their segment of homology, data are emerging that several of the proteins are involved in endocytosis and or regulation of cytoskeletal organization. We have named this protein segment the ENTH domain, for Epsin N-terminal Homology domain, and hypothesize that it is a candidate for binding specific ligands and/or enzymatic activity in the cell.

Keywords: Af10; clathrin; cytoskeleton; Dap160; DPF; DPW; EH domains; endocytosis; Eps15; Epsin; homology; intersectin; MP90; NPF; Pan1; protein–protein interaction

The ENTH domain is a highly conserved amino terminal domain of ~140 amino acids that is found in a variety of proteins from numerous species. ENTH domain-containing proteins were first identified in several laboratories because their divergent carboxy-termini interact with Eps15 Homology (EH) domains (Chen et al., 1998; Yamabhai et al., 1998; Wendland & Emr, 1998). The EH domain has recently been described as a protein interaction module contained in proteins involved in endocytosis (Di Fiore et al., 1997) and regulation of the actin cytoskeleton (Wendland et al., 1998). To understand its function in yeast, EH domain-binding proteins have been isolated by two-hybrid screening with the EH domains from the yeast protein, Pan1p, an essential protein for normal organization of the actin cytoskeleton (Tang & Cai, 1996) and endocytosis (Wendland et al., 1996). The screen yielded the yeast homolog of AP180, a clathrin-assembly protein, and three uncharacterized open reading frames (ORFs) as candidate interacting proteins (Wendland & Emr, 1998). Not only did the yeast AP180 and ORF YDL161w proteins carry multiple copies of the putative EH ligand motif, asparagine-proline-phenylalanine (NPF), in their C-terminal regions (Salcini et al., 1997), but their N-termini were similar. A computer search of the yeast genome revealed two other proteins, ORFs YLR206w and YJR125c, with N-terminal sequences, which strongly resembled those carried by the YDL161w protein.

Simultaneous to the work in yeast, two other proteins bearing similar N-terminal sequences have been discovered in mammals. These proteins were isolated from rat and mouse through affinity purification (Chen et al., 1998) and cDNA expression screening with different EH domains (Yamabhai et al., 1998), respectively. The rat protein, termed Epsin, binds to the EH domains of Eps15, a substrate for the epidermal growth factor tyrosine kinase with three EH domains. Eps15 is involved in clathrin-mediated endocytosis (Fazioli et al., 1993; Tebar et al., 1996; Carbone et al., 1997; Benmerah et al., 1998), and has been observed localized in nerve terminals where clathrin-mediated endocytosis of synaptic vesicles occurs (Chen et al., 1998). The mouse protein, Intersectin binding protein 2 (Ibp2), binds to Intersectin, a novel protein that was identified in *Xenopus laevis* that contains two EH domains and five Src Homology (SH) domains in its N- and C-termini, respectively (Yamabhai et al., 1998). cDNAs encoding a human homolog of Intersectin (Guipponi et al., 1998) and a related protein in *Drosophila melanogaster* (Roos & Kelly, 1998), termed Dynamin associated protein of 160,000 molecular mass (Dap160), have been cloned recently.

Careful comparison of the primary structures of the proteins that interact with the EH domains of Pan1p, Eps15, and Intersectin, revealed that they share a ~140 amino acid conserved segment. This segment, first noted in a plant (Oat) protein (Jones & Hooley, 1997), occurs in 12 proteins currently in GenBank, in such diverse genomes as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Rattus norvegicus*, *Mus musculus*, *Xenopus laevis*, *Avena fatua*, and *Homo sapiens*. The alignment of this

Reprint requests to: Brian K. Kay, University of Wisconsin-Madison, 1300 University Avenue, Madison, Wisconsin; e-mail: bkkay@facstaff.wisc.edu.

novel domain is shown in Figure 1. The primary structural identity among the sequences ranges between 42 and 93%, with 16 absolutely conserved residues, N-x_{11-13}-V-x_2-A-T-x_{34-36}-R-x_{7-8}-W-R-x_3-K-x_{12}-G-x-E-x_{15}-L-x_{11-12}-D-x-G-R-x_{11}-D-x_7-R. Interestingly, this conserved segment is present at or near the N-terminus of most proteins, even though their C-terminal sequences vary. Computer algorithms suggest that the domain is mostly α-helical in secondary structure and lacks any transmembrane spanning regions. We have named this conserved protein segment the "ENTH" domain, for Epsin N-Terminal Homology domain, after Epsin, 1 of the 12 proteins.

In GenBank searches, the only characterized protein with a region that weakly matches (22% similarity) the ENTH domain is the clathrin assembly protein, AP180 (also known as AP-3, NP185, pp155, F1-20). Interestingly, 9 of the 16 residues absolutely conserved in the ENTH domain are also conserved between the mouse,

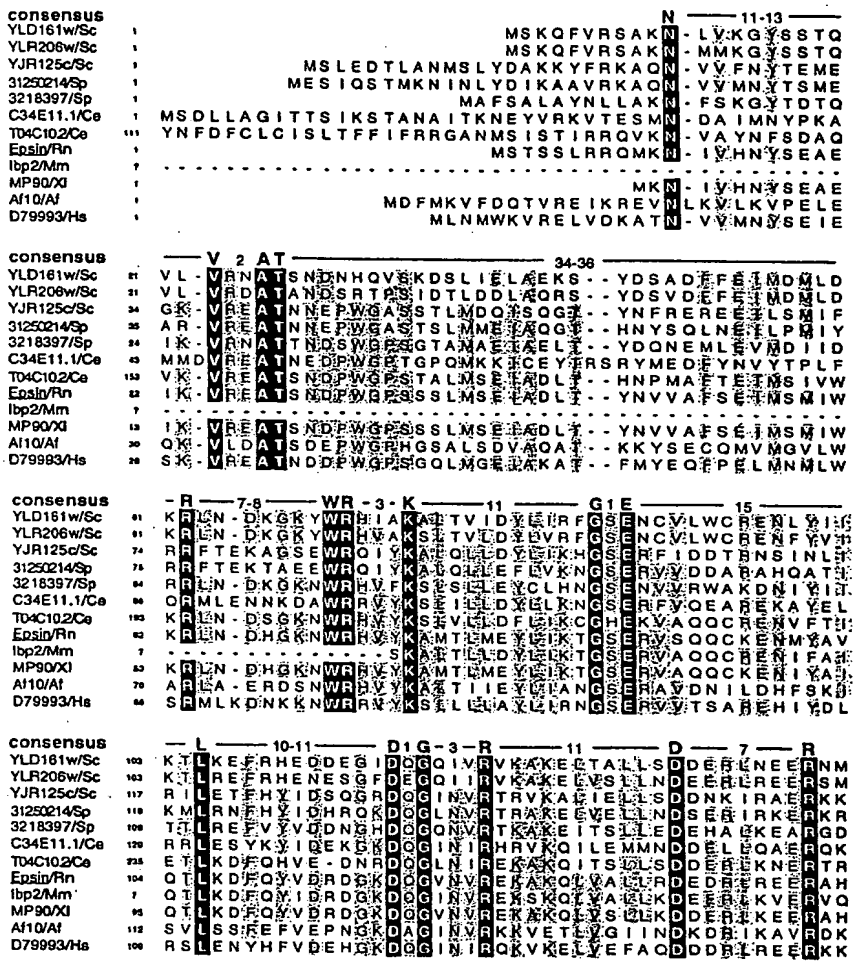

Fig. 1. Alignment of the primary structures of the ENTH domains in 11 proteins. The domains were aligned with ClustalW (http://www2.ebi.ac.uk/clustalw/) and the figure was generated with SeqVu (v1.1). The amino acid positions are numbered on the left. Gaps have been introduced in some of the sequence to maximize their alignment. Identical residues shared by ≥50% of the 12 entries have been highlighted; residues shared by all the entries are highlighted in black. The absolutely conserved residues, along with the number of intervening amino acids, are shown in the consensus. All sequences are full-length, except for Ibp2 which is missing its N-terminus. Sc, Sp, Ce, Rn, Mm, Xl, Af, and Hs abbreviations correspond to *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Rattus norvegicus*, *Mus musculus*, *Xenopus laevis*, *Avena fatua*, and *Homo sapiens*, respectively. The GenBank accession numbers are listed on the left column, except for Epsin, Ibp2, MP90, and Af10 which are 3249559, 3063649, 2072301, and 1724114, respectively. Further descriptions regarding some of the proteins, YLD161w (Wendland et al., 1998), MP90 (Stukenberg et al., 1997), Epsin (Chen et al., 1998), Ibp2 (Yamabhai et al., 1998), and Af10 (Jones & Hooley, 1997), can be found elsewhere.

human, and yeast AP180 proteins (Wendland & Emr, 1998); more specifically, these are N-x_{11-13}-V-x_2-A-T-x_{34-36}-R-x_{7-8}-W-R-x_3-K-x_{12}-G-x-E-x_{15}-L-x_{11-12}-D-x-G-R-x_{11}-D-x_7-R (matching residues are underlined). This region of similarity is contained within a larger segment (N-terminal 33 kDa) of AP180 previously shown to bind polyphosphoinositides and inhibit clathrin assembly (Ye & Lafer, 1995; Hao et al., 1997). This segment includes a motif implicated in the binding of phosphatidylinositol 3,4,5 trisphosphate by centaurin-α (Hammonds-Odie et al., 1996). While this motif falls within the region of AP180 similar to the ENTH domain, the consensus residues for lipid binding are not conserved in the ENTH domain.

Figure 2 diagrams the relative locations of the ENTH domain and other short motifs within the 12 proteins. Based on the biochemical and genetic characterization of only a few of the proteins, we postulate that these proteins are likely to function in endocytosis and/or regulation of the actin cytoskeleton. Ten of the 12 proteins contain sequences related to the clathrin-binding motifs described in arrestin (Krupnick et al., 1997), adaptor protein AP-3 (Dell'Angelica et al., 1998), and amphiphysin II (Ramjaun & McPherson, 1998). The proteins share either the motif L(L,I,V)(D,E,G,N)(L,F)(D,E,Q) or the related sequence, LIDL-COO^-. Interestingly, the rat Epsin protein, which carries the motif LVDLD, has recently been reported to bind clathrin (Chen et al., 1998). Many of the 12 proteins also carry the motif asparagine-proline-phenylalanine (NPF) in their C-terminal regions. This motif appears to be the ligand for the EH domains of Eps15 (Salcini et al., 1997), Pan1p (Wendland & Emr, 1998), and Intersectin (Yamabhai et al., 1998). Finally, many of the proteins also carry multiple copies of the tripeptide sequences DPF or DPW, even within the ENTH domain itself (i.e., C34E11.1, T04C10.2, MP90, epsin, D79993). At present, it is unclear what function(s) these tripeptides serve.

In conclusion, we propose that the ENTH domain is an important domain within proteins involved in endocytosis and the cytoskeleton. The amino acid conservation among the ENTH domains in proteins of fungal, plant, and animal origin strongly suggests that this domain is functionally important. Future experiments will determine whether or not the ENTH domain has ligand binding or enzymatic activity in the cell.

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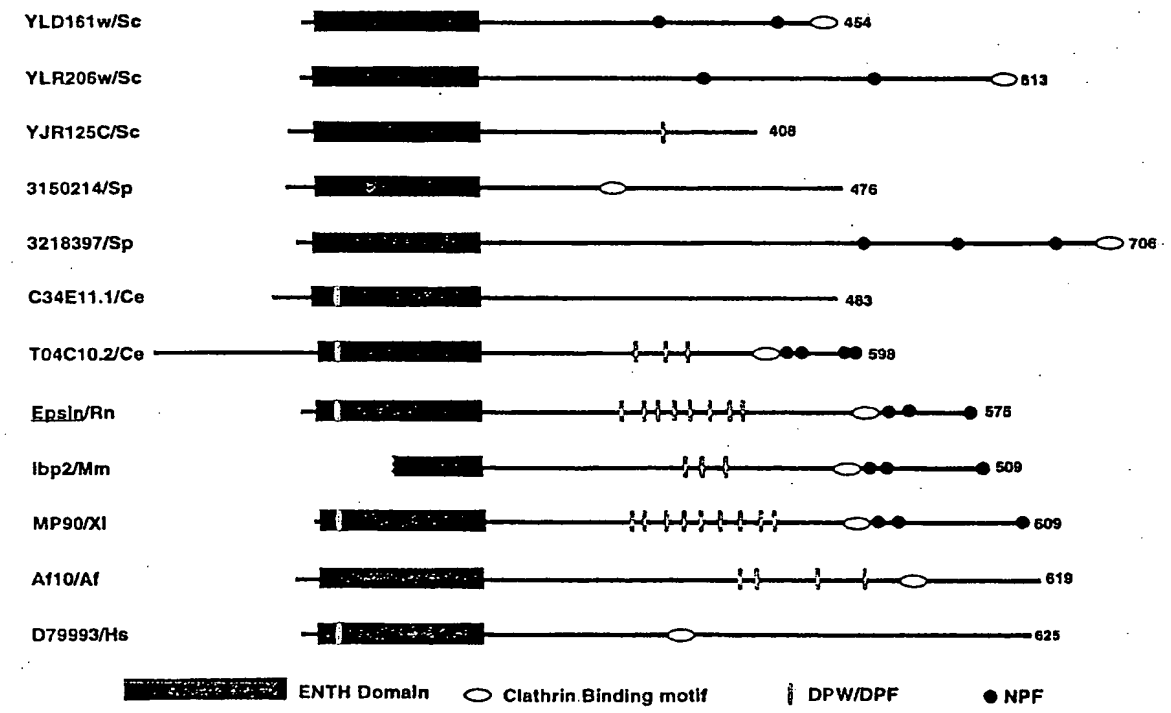

Fig. 2. Diagram of the proteins described in Figure 1. The ENTH domain is shown as a solid box. The proteins are referred to by their GenBank accession name, source, and length in amino acids. Sc, Sp, Ce, Rn, Mm, Xl, Af, and Hs abbreviations correspond to *S. cerevisiae*, *S. pombe*, *C. elegans*, *R. norvegicus*, *M. musculus*, *X. laevis*, *A. fatua*, and *H. sapiens*, respectively. The DPW/DPF, clathrin-binding, and NPF motifs are denoted by shaded bars, hollow ovals, and filled circles, respectively.

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The Epsins Define a Family of Proteins That Interact with Components of the Clathrin Coat and Contain a New Protein Module*

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Julie A. Rosenthal‡, Hong Chen‡, Vladimir I. Slepnev‡, Lorenzo Pellegrini‡, Anna Elisabetta Salcini§, Pier Paolo Di Fiore§¶, and Pietro De Camilli‡‖

From the ‡Howard Hughes Medical Institute and Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510, §Department of Experimental Oncology, European Institute of Oncology, Milan 20141, Italy, and ¶Unstituto di Microbiologia Universita' di Bari, Bari 70124, Italy

Epsin (epsin 1) is an interacting partner for the EH domain-containing region of Eps15 and has been implicated in conjunction with Eps15 in clathrin-mediated endocytosis. We report here the characterization of a similar protein (epsin 2), which we have cloned from human and rat brain libraries. Epsin 1 and 2 are most similar in their NH_2-terminal region, which represents a module (epsin NH_2 terminal homology domain, ENTH domain) found in a variety of other proteins of the data base. The multiple DPW motifs, typical of the central region of epsin 1, are only partially conserved in epsin 2. Both proteins, however, interact through this central region with the clathrin adaptor AP-2. In addition, we show here that both epsin 1 and 2 interact with clathrin. The three NPF motifs of the COOH-terminal region of epsin 1 are conserved in the corresponding region of epsin 2, consistent with the binding of both proteins to Eps15. Epsin 2, like epsin 1, is enriched in brain, is present in a brain-derived clathrin-coated vesicle fraction, is concentrated in the peri-Golgi region and at the cell periphery of transfected cells, and partially colocalizes with clathrin. High overexpression of green fluorescent protein-epsin 2 mislocalizes components of the clathrin coat and inhibits clathrin-mediated endocytosis. The epsins define a new protein family implicated in membrane dynamics at the cell surface.

Epsin (epsin 1) is a recently characterized protein with a putative role in clathrin-mediated endocytosis. Its COOH-terminal region, which contains three repeats of the EH domain binding consensus NPF, interacts with the EH domains of Eps15 (1–3), whereas its central region binds the clathrin adaptor AP-2 (4). Epsin 1 is enriched in brain and is partially associated *in situ* with clathrin coats. Disruption of epsin 1 function in fibroblasts by either overexpression, microinjection of its AP-2 binding central region, or microinjection of antibodies has a potent inhibitory effect on clathrin-mediated endocytosis (5).

Screening of a prokaryotic expression library from human fibroblasts with a GST[1] fusion protein comprising the EH domains of Eps15 led to the isolation of several clones interacting with these domains. One of them, EHB21 (6), encoded a partial sequence similar (34%) to the COOH-terminal region of rat epsin 1. Based on the EHB21 sequence, we have screened human and rat libraries to obtain full-length open reading frames. The sequences obtained encode a novel protein, epsin 2, which is closely related to but is distinct from epsin 1. We report here a characterization of this protein and demonstrate that epsin 2, like epsin 1, is implicated in clathrin function.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies against epsin 2 were directed against the coding amino acids of the EHB21 clone (6). Rabbit polyclonal antibodies Eps15 and epsin 1 were previously described (2, 5). Mouse monoclonal antibodies directed against clathrin (TD.1 and X.22), α-adaptin, and β-adaptin were obtained from ATCC (Manassac, VA), Affinity Bioreagents (Golden, CO), and Sigma, respectively. Anti-glutamic acid decarboxylase antibodies and anti-synapsin 2 antibodies were as described (7, 8)

Cloning of Epsin 2 cDNA—A 485-base pair fragment of EHB21 (6) comprising its coding region and the first 120 base pairs of the 3′-untranslated region was amplified by PCR from the clone using the following oligonucleotide primers: forward primer, 5′-ctggactcactggtgacc-3′; reverse primer, 5′-gctggagctgaaaagccc-3′. This fragment was radioactively tagged by [α-^{32}P]dCTP via random prime-labeling (Roche Molecular Biochemicals). The labeled product was used as a probe to screen a human cerebellar cDNA λZAP library (Stratagene, La Jolla, CA). A partial clone isolated from the screen (containing a 1 kilobase insert) was in turn used as a probe to screen a human brain λgt11 cDNA library (CLONTECH, Palo Alto, CA). Multiple screens of the λgt11 human brain library led to several overlapping clones, none of which contained a full-length open reading frame. A full-length clone (17/4) was assembled from clone 4, which contained the termination codon, and from clone 17B, which included the putative start codon ATG (within an apparent Kozak consensus) (8) but lacked the 3′ end. Clone 17/4 was generated by ligation of two gel-purified fragments (1151 and 1086 base pairs, respectively) obtained from digestion of clone 17 and clone 4 within pBluescript with *Eco*RI and *Mlu*I. The resulting construct was subcloned into pBluescript (epsin 2a/pBSK). The sequence of several other partial clones was identical to that of clone 17/4, henceforth defined as epsin 2a, with the exception of a 174-base pair deletion (see Fig. 1A), resulting in a 58-amino acid deletion in the open reading frame (epsin 2b). Nucleotide sequences were analyzed using the Lasergene software package (DNASTAR Inc., Madison, WI). The Genetics Computer Group implementation of Paup version 4.0d55 was used to generate a single maximum parsimony tree from the alignment shown in Fig. 2A. The phylogenetic tree is unrooted with bootstrap values

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF062085 (human 2a), AF062084 (human 2b), and AF096296 (rat 2).

‖ To whom correspondence should be addressed: Dept. of Cell Biology, Howard Hughes Medical Institute, 295 Congress Ave., New Haven, CT 06510. Tel.: 203-737-4465; Fax: 203-737-1762; E-mail: pietro.decamilli@yale.edu.

[1] The abbreviations used are: GST, glutathione *S*-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein.

indicated along the branches to assess the confidence of each branch of the topology. All branches are resolved with a high (>50%) confidence (33).

The rat epsin 2 sequence was obtained by PCR cloning from a rat brain cDNA library using primers that were generated based on human epsin 2 sequences. Three pairs of primers ((5'-GCAAGGACCAGGGCATCAATG-3', 5'-TGAAGTCCGAAGGTTGTCAAATTC-3') and (5'-CCCCGGCCTTGCCATACT-3', 5'-CAGCCACTCGGAGCAGGAGTAT-3'), and (5'-ATGACAACTTCGTCTATCAGACGG-3', 5'-CTAGAGAAGGAAAGGGTTGGTTGTG-3')) were added to a single PCR at a final concentration of 2 mM. The longest PCR product was gel-extracted and subcloned into a TA vector (Invitrogen) and fully sequenced, confirming the generation of a full-length rat epsin 2 clone.

Production of Recombinant Proteins—A portion of the coding region of human epsin 2 containing the three NPF motifs (amino acids 524–642 human epsin 2a), the DPW domains of epsin 2 (amino acids 318–456 of human epsin 2a), and the ENTH/DPW domain of epsin 2 (amino acids 1–456 of human epsin 2a) and epsin 1 (amino acids 1–401) were amplified by PCR using *Taq* polymerase. The PCR fragment encoding the NPF motifs was subcloned into the TA vector (Invitrogen, Carlsbad, CA), cleaved with *Eco*RI, and ligated to pGEX4T-2 (Amersham Pharmacia Biotech) to obtain a GST-NPF fusion protein. The PCR product of the DPW domain of epsin 2 was digested with *Eco*RI/*Xho*I and subcloned into the same vector to obtain a GST-DPW fusion protein. The ENTH/DPW domain fragments were digested with *Eco*RI/*Xho*I (epsin 1) and *Hin*dIII/*Xho*I (epsin 2) and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). GST fusions of the ENTH/DPW of epsin 1 and 2 were prepared by subcloning *Eco*RI/*Xho*I inserts into pGEX6 (Amersham Pharmacia Biotech). DH5α or BL21(DE3) host strains were transformed, and both constructs were verified by sequencing (Keck Biotechnology Resource Laboratory, Yale University). Rat epsin 1 constructs and GST fusion proteins of amphiphysin 1 (amino acids 262–405 and 262–375) were previously described (5, 9). Proteins were purified on a glutathione 4B-Sepharose affinity matrix (Amersham Pharmacia Biotech).

Affinity Chromatography—Affinity chromatography of a rat brain extract on the GST-NPF domain of epsin 2 was performed as described previously (9). For the study of AP-2 and clathrin binding to GST fusion proteins, a rat brain homogenate was extracted with 1 M Tris, pH 8.9, 2% Triton X-100, and a protease inhibitor mixture and spun at 100,000 × *g* for 1 h. The supernatant was desalted in a buffer containing 125 mM KCl, 25 mM Hepes, and 1% Triton X-100. Proteins specifically retained by the affinity matrix were eluted into SDS/PAGE sample buffer, separated by SDS/PAGE, and transferred to nitrocellulose for Western blotting (9).

In Vitro Translations—cDNA clones encoding luciferase (Promega Co.), amphiphysin I (10), rat epsin 1 (5), human epsin 2, and fragments of rat epsin 1 and human epsin 2 comprising both the ENTH and DPW domains (amino acids 1–401 and 1–456, respectively) were *in vitro* transcribed and translated using [^{35}S]methionine with a T7-coupled transcription/translation system (Promega Co.) according to manufacturer's instructions. The radiolabeled products were separated by SDS/PAGE and quantitated so that equivalent amounts of radioactivity could be added in each binding reaction. The products were then incubated for 2 h at 4 °C in phosphate-buffered saline containing 1% Triton X-100, 5 mM EDTA, and 1 mg/ml bovine serum albumin with 50 μl of glutathione-Sepharose with a prebound GST or GST fusion protein of the NH_2-terminal 579 amino acids of the clathrin heavy chain (kindly provided by James Keen, Kimmel Cancer Institute). The beads were pelleted by centrifugation and thoroughly washed in the same buffer. The bound proteins were eluted in 40 μl of SDS sample buffer and separated by SDS/PAGE followed by audioradiography.

Subcellular Fractionation—Subcellular fractionation of rat brain to obtain a fraction enriched in clathrin-coated vesicles was performed as described (11).

Cell Transfections and Immunofluorescence—A full-length GFP-tagged human epsin 2a clone was constructed by PCR modification of the 5'-end of the epsin 2a sequence to incorporate additional restriction sites. This clone was subcloned into an enhanced green fluorescent protein vector with *Xho*I/*Bam*HI. Full-length Xpress-tagged rat epsin 1 was prepared as described (5). The cDNA-containing vectors were purified with a Maxiprep kit (Qiagen, Chatsworth, CA) and used to transfect Chinese hamster ovary cells by the LipofectAMINE method (Life Technologies, Inc.). Transfected cells were fixed in 4% paraformaldehyde and subjected to immunofluorescent staining as described (12).

RESULTS

Domain Structure and Expression Pattern of Epsin 2—A construct derived from the EHB21 clone was used as the initial probe to isolate via sequential library screens several overlapping human DNA sequences that encode an open reading frame, epsin 2, closely related to epsin 1. A corresponding full-length clone (epsin 2a) was assembled from two overlapping clones encoding the 5' (clone 17B) and 3' ends (clone 4) of the open reading frame. The putative start codon is surrounded by a sequence that conforms to the initiation consensus sequence as defined by Kozak (13) and is preceded by an in-frame stop codon. Furthermore, it aligns with the start ATG of epsin 1. Other clones encoded identical sequences, with the exception of a deletion of amino acids 200–257, present in the majority of clones. This finding suggests the occurrence of a shorter epsin isoform (epsin 2b) resulting from alternative splicing. The presence of the two epsin 2 variants was confirmed by PCR amplification from a human brain library of the portion of the epsin 2 gene comprising this region (data not shown).

A PCR-based strategy was then used to obtain from a rat brain library the nucleotide sequence of rat epsin 2. The sequence obtained does not include the 58-amino acid insert (see above). The deduced amino acid sequences of human and rat epsin 2 (accession numbers: AF062085 (human 2a), AF062084 (human 2b), and AF096296 (rat 2)) are shown in Fig. 1*A*, where they are aligned with each other and with rat epsin 1. Epsin 2 comprises the same main domains as epsin 1, with the NH_2-terminal portion of the protein, which contains the epsin homology domain (5) (epsin NH_2-terminal homology: ENTH) being the most conserved between rat epsin 1 and 2 (~85% identity) and between human and rat epsin 2 (~98% identity) (Fig. 1*B*). This domain is present in a variety of partial and complete open reading frames from several species in the data base (Fig. 2*A*), including open reading frames otherwise substantially dissimilar from epsin. It appears, therefore, to be a protein module with a conserved function from yeast to man (Fig. 2). As shown by the alignment of Fig. 2*A*, a few amino acids of the ENTH domain are 100% conserved in all of the sequences.

The carboxyl-terminal region (NPF domain) is less conserved between epsins 1 and 2 (33% identity) but contains three NPF motifs in both proteins. This is consistent with the isolation of EHB21 as an interactor for the EH domain of Eps15 (6). The DPW motifs of the central region (DPW domain) are only partially conserved between epsin 1 and epsin 2. This central region is the least conserved between rat epsin 1 and 2 (~26% identity) and also between rat and human epsin 2 (~79% identity) (Fig. 1*B*). The DPW domain of epsin 2 contains the sequence LLDL, which precisely fits the core of the putative consensus for binding to clathrin (14–16). The *Xenopus* mitotic phosphoprotein MP90 (17) is more similar to epsin 1 (~51%) than to epsin 2 (~40%), and the putative phosphorylation site for Cdc2 kinase in MP90 (17) is also present in the DPW domain of epsin 1 (5) but not in that of epsin 2.

In Western blots, antibodies generated against a GST-EHB21 fusion protein (corresponding roughly to the NPF-containing region of epsin 2) reacted primarily with a doublet of 74- and 65-kDa bands in a human brain homogenate and a 74-kDa protein band in a rat brain homogenate (Fig. 3*A*). The same antibodies recognized a 74-kDa band in Chinese hamster ovary cells transfected with hemagglutinin-tagged human epsin 2 (Fig. 3*B*). The 65-kDa band visible in human brain homogenate may therefore represent either an isoform or a proteolytic fragment of the 74-kDa band. A 74-kDa apparent molecular mass is only slightly higher than the predicted size of epsin 2 band based on its amino acid composition (68 kDa for

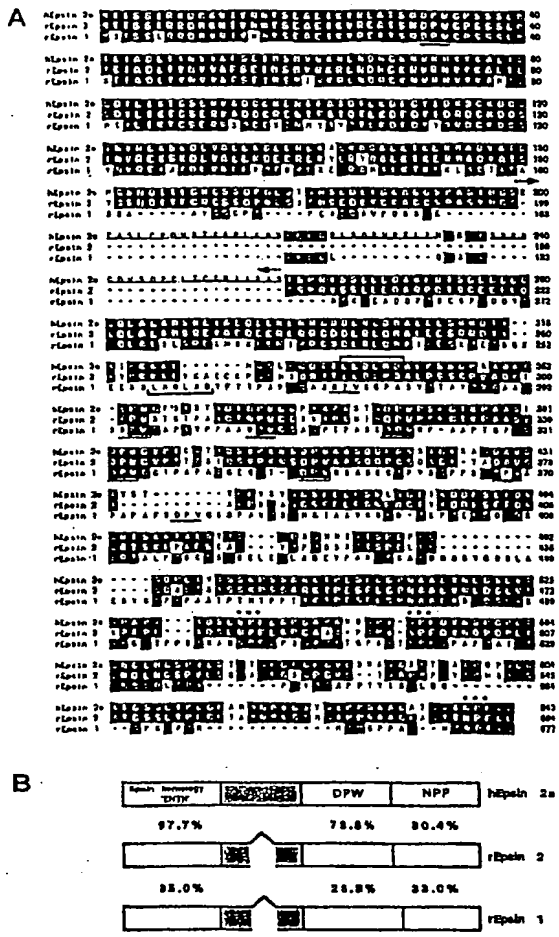

FIG. 1. Alignment of the deduced amino acid sequence of human (*h*) epsin 2a, rat (*r*) epsin 2, and rat epsin 1. *A*, primary amino acid sequence of the three proteins. A segment that is absent in human epsin 2b is indicated by *arrows*. DPW motifs are *underlined*, and NPF motifs are indicated by *asterisks*. The sequences that share similarity to a clathrin binding consensus in the clathrin binding fragments of epsin 1 and 2 are indicated by *brackets*. *B*, domain structure of the three proteins. *Numbers* indicate the percent identities between corresponding domains.

human epsin 2a). In contrast, epsin 1, whose predicted size is only slightly greater than that of epsin 2, migrates with an apparent molecular mass of about 94 kDa in SDS/PAGE (Fig. 3*A*). Based on the analysis of the SDS/PAGE mobility of epsin fragments, this difference is primarily due to the different mobilities of the DPW domains of epsin 1 and 2 (not shown). It is most likely due to the higher content of acidic amino acids in the DPW domain of epsin 1 (predicted pI 3.3) than in the DPW domain of epsin 2 (predicted pI 9.08). As shown in Fig. 3*B*, antibodies directed against epsin 2 do not recognize epsin 1 in either brain or transfected cells.

Western blot analysis of a variety of rat and human tissues demonstrated a widespread tissue distribution of the protein (Fig. 4). Similar results were obtained by Northern blotting (not shown). The highest concentration of the protein was observed in brain.

Epsin 2 Binds Eps15, α-Adaptin, and Clathrin—We next investigated the binding properties of epsin 2. Extracts of rat brain were affinity-purified on GST fusion proteins of epsin 2 fragments, and the bound proteins were revealed by Western blotting. The NPF domain bound specifically to Eps15 (Fig. 5) but not to glutamic acid decarboxylase, another abundant brain cytosolic protein (18) used as a control, in agreement with the isolation of the EHB21 clone as an interactor for its EH domains (6).

The DPW domain of epsin 2 bound AP-2, as revealed by the enrichment in the bound material of the AP-2 subunits, α- and β-adaptins (3) (Fig. 6). Blotting of the material affinity-purified by this construct for a variety of endocytic proteins demonstrated that the DPW domain of epsin 2 also bound clathrin (Fig. 6*B*), in agreement with the presence of the clathrin binding motif LLDL in this domain (14–16). GST, used as a negative control, did not bind either protein, whereas amphiphysin 1, used as a positive control, bound both AP-2 and clathrin as expected (Fig. 6, *A* and *B*). A GST fusion protein of the DPW domain of epsin 1 bound clathrin only slightly above background (not shown). We noted, however, that the sequence LMDLAD is present at the very NH_2-terminal end of this construct of epsin 1, and unfavorable folding of this fusion protein may interfere with binding to clathrin. We considered the possibility that epsin 1 as well may bind clathrin and that flanking regions may be required for optimal binding of clathrin to epsin. We compared, therefore, binding of GST fusion proteins of longer epsin 1 and epsin 2 constructs (amino acids 1–401 of epsin 1 and amino acids 1–456 of epsin 2). As shown by Fig. 6*C*, both constructs, but not GST alone, bound similar levels of AP-2 and similar levels of clathrin. Clathrin binding was also observed when the brain extract used for the affinity purification was depleted of AP-2 by preincubation with a GST fusion protein comprising selectively the AP-2 binding domain of amphiphysin 1 (10) (not shown). This finding strongly suggests that not only the interaction between epsin and AP-2 (5), but also the interaction between epsin and clathrin, is direct.

To further demonstrate a direct interaction between clathrin and epsin 1 or 2, *in vitro* translation experiments were performed. Epsin 1 and epsin 2 fragments corresponding to those used for Fig. 6*C*, amphiphysin 1 (positive control) and luciferase (negative control), were transcribed and translated *in vitro* and then incubated with immobilized GST or a GST fusion of the NH_2-terminal domain of clathrin. This clathrin domain was previously shown to be the region of clathrin that binds the consensus sequence L(L/I)(D/E/N)(L/F)(D/E) (14, 19). As shown by Fig. 7, *epsin 1* and *epsin 2*, their ENTH-DPW domain fragments and amphiphysin 1 bound clathrin, whereas luciferase did not. None of the proteins bound GST alone (not shown). Binding of epsin 1 and 2 to clathrin was similar to that of amphiphysin 1 in this assay.

Epsin 2 Is Partially Associated with Clathrin Coats—Rat brain homogenate was fractionated to generate a highly enriched clathrin-coated vesicle fraction. Epsin 2 was partially recovered in the purified coated vesicles, although it did not co-enrich with clathrin and AP-2, *i.e.* the intrinsic components of the clathrin coat. Instead, its recovery in the clathrin-coated vesicle fraction was similar to that of Eps15 (Fig. 8), epsin 1, and of other accessory proteins of the clathrin coat (5) (Fig. 8). Synapsin 2, a protein previously found to be absent from clathrin-coated vesicles (8) was not present in the same fraction.

The subcellular localization of epsin 2 was further analyzed in cultured fibroblastic cells. Since endogenous epsin 2 was not detectable by available antibodies, epsin 2 was expressed in

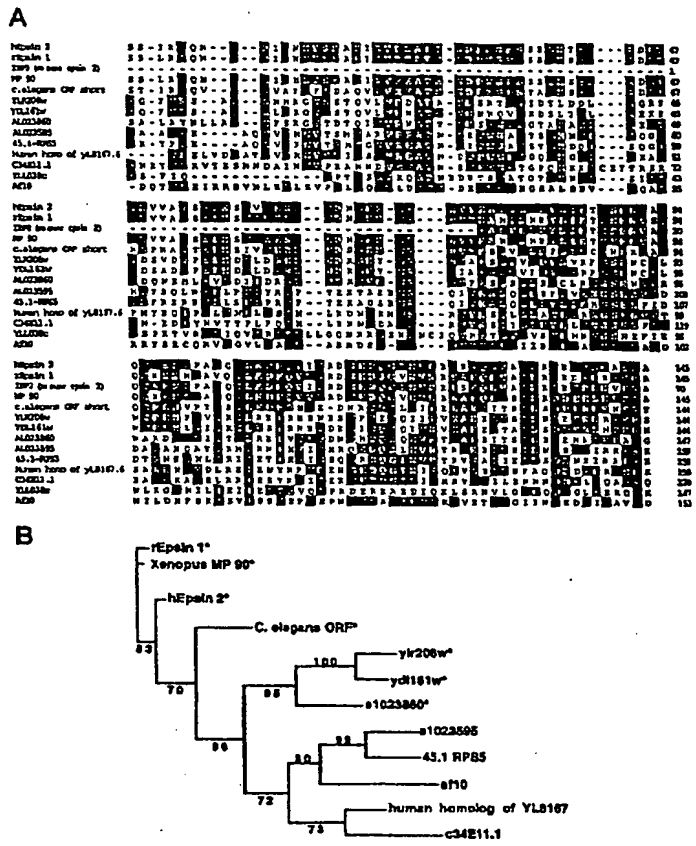

FIG. 2. The NH_2-terminal region of epsin 2 defines an evolutionary conserved protein module (ENTH domain). *A*, alignment of ENTH domains from a number of proteins in the data base produced by the Clustal program in Lasergene. *B*, phylogram illustrating the evolutionary relationship of these domains using the Genetics Computer Group implementation of Paup version 4.0d55 (see methods). The sequence of Ibp2 (which is only a partial sequence) was not included (20). *Asterisks* indicate proteins that also contains NPF motifs. *h*, human; *r*, rat; *C. elegans*, *Caenorhabditis elegans*; *ORF*, open reading frame;

these cells as a GFP-tagged fusion protein. In cells expressing it at low to moderate levels, GFP-epsin 2 appeared as fine puncta sparse throughout the cell and particularly concentrated in the central region where the Golgi complex is localized. This distribution was very similar to the distribution of clathrin immunoreactivity (Fig. 9, *a* and *b*). In highly expressing cells (Fig. 9, *c–j*), GFP-epsin 2 produced an intense and continuous labeling of the cell surface as well as one or more bright, compact masses in the central region of the cell. In these cells a major redistribution of components of the clathrin coat was observed, as shown by the collapse of clathrin, Eps15, and to a lesser extent, AP-2, into the same central mass(es) positive for GFP-epsin 2 (Fig. 9, *c–h*). Furthermore, in these cells, an inhibition of the internalization of Texas red-labeled transferrin was observed (Fig. 9, *i* and *j*). Similar results were obtained by using epitope-tagged epsin 2 instead of GFP-epsin 2 (data not shown).

DISCUSSION

We report here the characterization of a new protein that shares significant primary sequence similarity with epsin (epsin 1) and that we have therefore defined as epsin 2. Epsin 1 and 2 have the same tripartite domain structure, similar protein binding partners, and similar subcellular localization. Furthermore, both proteins are enriched in brain. Despite these similarities, a number of differences have also emerged.

The region of highest similarity between the two proteins is the NH_2-terminal domain, most strikingly, the first 150 amino acids. Based on data base searches, this domain defines a new protein module conserved from yeast to man (5) (epsin NH_2-terminal homology domain or ENTH domain). Alignment of these sequences reveals several sites with 100% conservation and numerous other sites with conservative amino acid substitutions. It is of interest that about half of these proteins and perhaps more, due to the incomplete sequence of some of these genes, contain one or multiple NPF motifs, even if some of them do not appear to represent true orthologues of either epsin 1 or 2. Since the NPF sequence was found to represent an EH domain binding consensus, this observation suggests that a physiologically important link between ENTH domains and EH domain-mediated interactions. This protein module, first identified in epsin 1 (5), has been recently and independently discussed by Kay *et al.* (20).

The second most conserved portion in epsin 1 and 2 is the COOH-terminal region, which contains the three NPF motifs. The interaction of this region with Eps15, predicted by the expression screen that led to the isolation of the EHB21 clone, was now confirmed by affinity chromatography of Eps15. The least conserved region is the central DPW domain, which contains only 3 fully conserved DPW repeats in epsin 2, in contrast to 8 repeats in the corresponding domain of epsin 1. Most important, although this domain is very acidic in epsin 1, it has a basic isoelectric point in epsin 2. This difference correlates

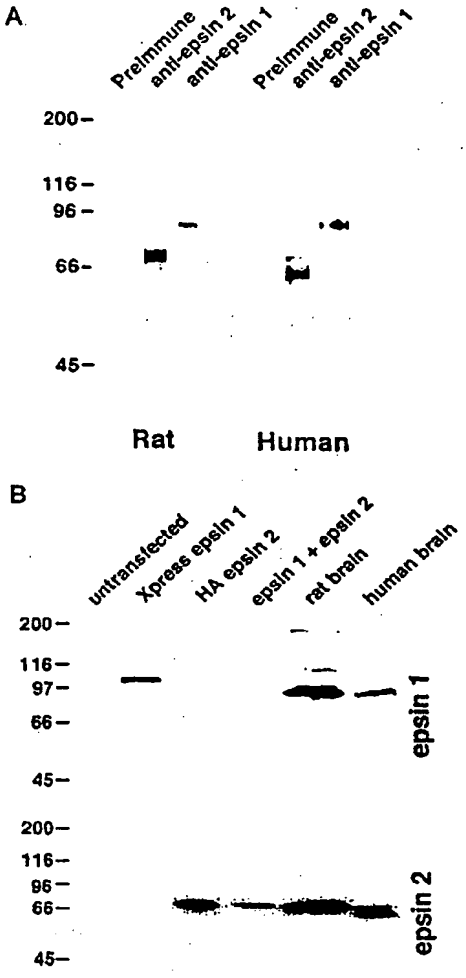

FIG. 3. Electrophoretic mobility of epsin 1 and 2 in transfected cells and brain. *A*, Western blot of rat and human brain homogenate using preimmune, anti-epsin 1, or anti-epsin 2 antisera. *B*, Western blots of Chinese hamster ovary cell lysates transfected with the indicated epsin constructs and human and rat brain homogenates. *HA*, hemagglutinin.

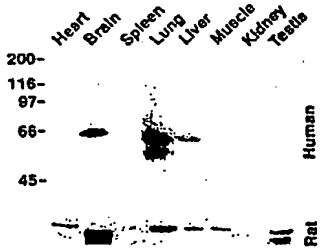

FIG. 4. Tissue distribution of epsin 2. Western blot analysis of the pattern of expression of epsin 2 in human and rat tissues.

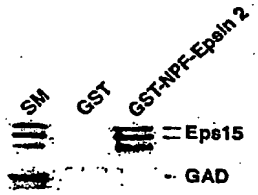

FIG. 5. The NPF domain of epsin 2 binds Eps15. A Triton X-100 rat brain extract was affinity-purified on GST or a GST fusion protein comprising the three NPF motifs of epsin 2. The starting material (*SM*) and the bound material were reacted by Western blotting for Eps15 or, as a control protein, the abundant cytosolic brain protein, glutamic acid decarboxylase.

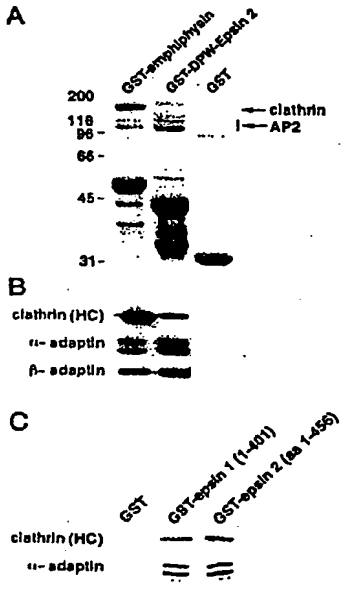

FIG. 6. The DPW domains of epsin 1 and 2 bind clathrin and AP-2. *A* and *B*, a Triton X-100 extract of rat brain was affinity-purified on similar amounts of GST or GST fusion proteins comprising amino acids 262–405 of amphiphysin 1 or the DPW domain of epsin 2 (amino acids 318–456). The bound material was stained by Coomassie Blue (*A*) or reacted by Western blotting for the heavy chain of clathrin and for the AP-2 subunits α- and β-adaptins (*B*). Note that *lanes* corresponding to GST were from the same gel as other samples. *C*, Western blotting for the heavy chain (*HC*) of clathrin and for α-adaptin of the material affinity-purified by GST fusion proteins of epsin 1 and 2 fragments comprising both the ENTH and the DPW domains.

with the presence of a phosphorylation site for Cdc2 kinase in epsin 1 (5), but not in epsin 2, and with corresponding observation that epsin 1, but not epsin 2, undergoes a phosphorylationdependent shift in electrophoretic mobility in mitotic cells (5, 17, 21).

Despite these differences, both DPW domains have similar AP-2 binding properties. Thus, the peculiar and distinct organization of the DPW motifs in the two proteins must have additional functions beside AP-2 binding. However, a contribution of at least some DPW repeats to AP-2 binding is plausible. It was shown previously that the unique arrangements of the multiple DPF motifs in the COOH-terminal region of Eps15 is not essential for AP-2 binding, although some of the numerous

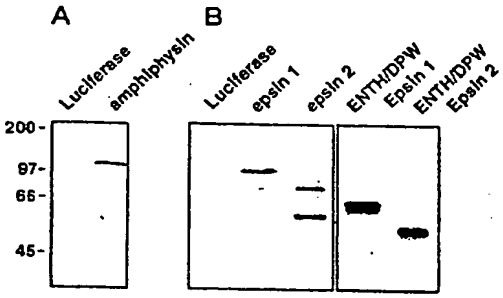

FIG. 7. Epsins 1 and 2 directly bind clathrin. The ^{35}S-labeled products of *in vitro* transcribed and translated cDNAs encoding luciferase, amphiphysin I, rat epsin 1, human epsin 2a, ENTH-DPW epsin 1 (amino acids 1–401), ENTH-DPW epsin 2a (amino acids 1–456) were incubated with immobilized GST fusion protein comprising the NH_2-terminal 579 amino acids of clathrin heavy chain. Bound proteins were visualized by autoradiography. A *lower band* in *epsin 2 lane* denotes a fragment of epsin 2 that likely results from a premature termination of the translation product, as this lower protein does not bind the EH domain of Eps15.

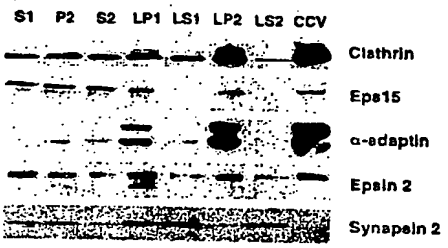

FIG. 8. Epsin 2 is present in a purified clathrin-coated vesicle fraction. Subcellular fractions of rat brain obtained during the preparation of clathrin-coated vesicles (*CCV*) (11) were reacted by Western blotting for the proteins indicated. Note that the fractions are defined according to Maycox *et al.* (11) and refer to fractions isolated in the preparation of clathrin-coated vesicles and that the LP2 fraction differs from that described by Huttner *et al.* (8) in the preparation of synaptic vesicles.

DPF motifs were found to participate in such an interaction (1, 22).

Both epsins bind the NH_2-terminal domain of clathrin. Epsin 1 and 2 contain the sequences LMDLAD and LLDLMD, respectively, at the NH_2-terminal side of their DPW domains. Based on the similarity of these sequences to a previously defined consensus for clathrin binding (L(L/I)(D/E/N)(L/F)(D/E)) (14, 15), it is conceivable that such sequences may represent the core of the clathrin binding domain.

All these findings support the hypothesis that epsin 2, like epsin 1, is implicated as an accessory factor in clathrin-mediated endocytosis. This hypothesis is further confirmed by the partial co-localization of GFP-epsin 2 with clathrin in transfected cells and by its property, when greatly overexpressed, to mislocalize clathrin coat components and to block the clathrin-mediated internalization of transferrin. In highly overexpressing cells, a pool of GFP-epsin 2 was concentrated under the plasmamembrane despite the coalescence of clathrin coat components and of the majority of GFP-epsin 2 to the central region of the cell. It was shown previously that epsin 1 as well, when overexpressed by transfection, can reach a concentration at the cell surface that far exceeds the concentration of clathrin, AP-2, and Eps15 (5). One must infer that epsin 1 and 2 have binding sites in the cortical cytomatrix besides components of the clathrin coat.

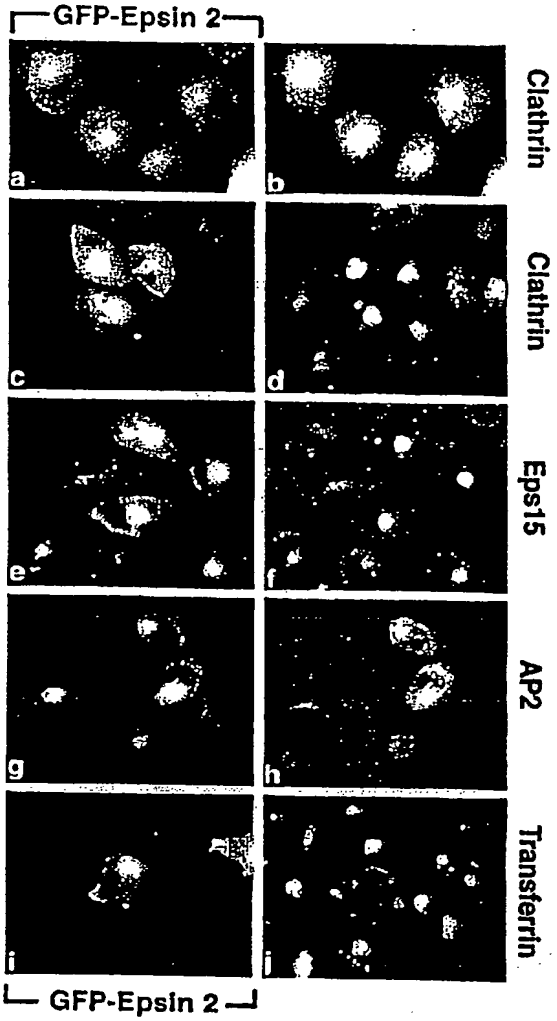

FIG. 9. Double fluorescence images showing the localization of GFP-epsin 2 in transfected Chinese hamster ovary cells and the effect of its overexpression on the localization of endocytic markers. GFP-epsin 2 colocalizes with clathrin (*a* and *b*) when expressed at a low concentration. In highly overexpressing cells (*c–e*), the localization of AP-2, clathrin, and Eps15 is severely disrupted. In these cells the internalization of transferrin is impaired (*h* and *i*).

The partial sequences of two proteins, Ibp1 and Ibp2, intersectin-binding proteins 1 and 2, which are likely to represent mouse epsin 1 and 2, respectively, were reported while this study was in progress (22). Intersectins (also referred to as Ese 1 and 2) (23) are the vertebrate homologues of the *Drosophila* DAP160 protein (24) and contain multiple SH3 domains and EH domains (25, 26). They bind epsin 1 (Ibp1) and 2 (Ibp2) via their NH_2-terminal EH domain and Eps15 via a coiled-coil region (22, 23). Since DAP160 and the intersectins also bind dynamin and synaptojanin (24), proteins of the DAP160/intersectin family may link the function of these proteins to the function of Eps15 and epsin. A picture is emerging in which

formation of a clathrin-coated vesicle is assisted by a variety of accessory factors that interact with each other to form large macromolecular complexes. At least one component of these complexes is a lipid-metabolizing enzyme, the inositol phosphatase synaptojanin (27). Other proteins of the complex have been linked to actin function from genetic studies in yeast and biochemical studies in mammalian tissues (28–31). It is therefore likely that this network of protein-protein interactions may coordinate formation of a coated bud and then of a free vesicle, with changes in the actin cytoskeleton and in the lipid components of the vesicle membrane.

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Addendum—While this paper was in revision a paper reporting the interaction of Ibp2 (epsin 2) with clathrin was reported (32).

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